

INFECTION AND IMMUNITY, May 1992, p. 1734–1739
0019-9567/92/051734-06\$02.00/0
Copyright © 1992, American Society for Microbiology

Vol. 60, No. 5

Characterization of a Neutralizing Monoclonal Antibody to *Pasteurella haemolytica* Leukotoxin

DONALD G. GERBIG, JR.,^{1†} MARK R. CAMERON,¹ DOUGLAS K. STRUCK,² AND ROBERT N. MOORE^{1*}

Department of Microbiology, University of Tennessee, Knoxville, Tennessee 37996,¹ and Department of Medical Biochemistry and Genetics, College of Medicine, Texas A & M University, College Station, Texas 77843²

Received 25 October 1991/Accepted 18 February 1992

Six hybridoma clones producing monoclonal antibodies (MAbs) reactive with *Pasteurella haemolytica* A1 leukotoxin were derived from mice immunized with leukotoxin excised from sodium dodecyl sulfate-polyacrylamide gels. Of the six MAbs, only one, Ltx-2, neutralized leukotoxin in a BL-3 cell cytotoxicity assay. MAb Ltx-2 blocked association of A1 leukotoxin to BL-3 cells, as measured by flow cytometric analysis. The epitope recognized by Ltx-2 was localized to the carboxyl half of the native protein, between residues 450 and 939, by Western immunoblot analysis of CNBr fragments. Further analysis with leukotoxin deletion proteins indicated either that the Ltx-2-reactive epitope was localized in the carboxyl portion of the leukotoxin between amino acids 768 and 939 or that this region influences MAb recognition of the epitope. MAb Ltx-2 was tested for neutralizing activity against leukotoxin produced by *P. haemolytica* serotypes 1 through 12. The MAb neutralized leukotoxin produced by all of the A biotype isolates (serotypes 1, 5, 6, 7, 8, 9, and 12), with the exception of serotype A2, but did not neutralize any T biotype leukotoxin tested (T3, T4, or T10). The results indicate that MAb Ltx-2 neutralizes leukotoxin by interfering with target cell association and that the MAb-specific epitope is either not present or not critical for function in the leukotoxin produced by *P. haemolytica* serotypes A2, T3, T4, and T10.

The ruminant-specific leukotoxin produced by *Pasteurella haemolytica* A1 is generally considered an important virulence determinant in the pathogenesis of bovine pneumonic pasteurellosis (1). Acting as a Ca^{2+} -dependent pore-former (6, 14), the toxin is believed to promote bacterial proliferation by killing or incapacitating phagocytes (12, 19). Moreover, leukotoxin-damaged phagocytes may contribute to the severe localized inflammatory response characteristic of this disease (34). Natural field resistance is associated with circulating titers of neutralizing antibodies (13), and such antibodies are detectable in lung fluids of calves protectively immunized by aerosol exposure to viable *P. haemolytica* A1 (29). In addition, recombinant leukotoxin has been used with measurable success as an experimental vaccine component (7).

The leukotoxin has been cloned and sequenced (20, 25, 26) and has significant homology with a variety of potential pore-forming toxins collectively characterized as the RTX toxin family (24, 35). Toxins in the RTX family have tandemly repeating glycine-rich sequences believed, at least for the *Escherichia coli* alpha-hemolysin, to serve as a functionally critical Ca^{2+} -binding region (3, 27). In addition, *P. haemolytica* leukotoxin shares with alpha-hemolysin an extended hydrophobic region containing a predicted membrane-spanning domain which is necessary to effect cytolysis (24, 30). The carboxy-terminal amino acids, at least for the alpha-hemolysin, appear to function in secretion (11), while the amino-terminal region appears to facilitate cytolysis, perhaps in the initial stages of host membrane insertion (37). Binding and cytolysis appear to be separable events (9, 27), and recent evidence indicates that lysis-defective mutant leukotoxin proteins with whole or partial deletions of the

membrane-spanning region retain the capacity to bind to target cells and to competitively inhibit cytolysis by native leukotoxin (8).

Monoclonal antibodies (MAbs) prepared against the alpha-hemolysin have helped to further define important domains within the toxin (22, 30), including a potentially important domain between the membrane-spanning region and the repeating glycine-rich domain that contains an epitope which, when bound by antibody, results in potent neutralization (31, 37). Similar investigations have not been performed with MAbs directed against *P. haemolytica* A1 leukotoxin.

We report here the production of murine MAbs raised against *P. haemolytica* A1 leukotoxin. One leukotoxin-neutralizing MAb which blocked association of the leukotoxin with sensitive target cells was identified. Further evaluation revealed that the epitope recognized by the neutralizing MAb was distributed or expressed differently in leukotoxins produced by different *P. haemolytica* serotypes and most notably was either absent or not critical for the toxic effects of leukotoxins from T biotypes and A2 serotypes. Preliminary epitope mapping indicated that the neutralizing antibody binds to a site located between residues 450 and 939 of the intact 953-residue native leukotoxin.

MATERIALS AND METHODS

Bacteria. The A1 *P. haemolytica* strain used to generate leukotoxin for the production of antibodies was isolate 88-1165, provided by R. D. Walker, Michigan State University, East Lansing, Mich. Additional *P. haemolytica* serotypes were obtained either from G. Frank, National Animal Disease Center, Ames, Iowa, or the American Type Culture Collection, Rockville, Md. These organisms were maintained on blood agar consisting of BHI agar (BBL, Cockeysville, Md.) and 5% defibrinated sheep blood, grown at 37°C

* Corresponding author.

† Present address: Department of Microbiology, SUNY at Buffalo, Buffalo, NY 14214.

(36). The organisms were stored in sterile skim milk at -70°C .

Cell culture. The BL-3 bovine leukemia-derived B-lymphocyte cell line used for the cytotoxicity assays was provided by G. H. Theilen, University of California, Davis. BL-3 cells were grown in RPMI 1640 medium (Sigma Chemical Company, St. Louis, Mo.) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, Utah), L-glutamine, and penicillin-streptomycin (Sigma) at 37°C in a humidified atmosphere of 5% CO_2 in air.

Leukotoxin production. Leukotoxin filtrates were prepared from log-phase *P. haemolytica* as described by Shewen and Wilkie (33). The supernatant culture medium was passed through a 0.2- μm -pore-size filter (Corning Glass Works, Corning, N.Y.) and concentrated 100-fold with a Pellicon concentrator equipped with a 30-kDa exclusion membrane (Millipore, Bedford, Mass.). The concentrated filtrate was aliquoted and stored at -70°C .

Cytotoxicity assay. Leukotoxin was measured by the neutral red cytotoxicity assay with BL-3 target cells, as reported by Greer and Shewen (16). Percent cytotoxicity was determined as the ratio of A_{540} in test wells with leukotoxin to A_{540} in control wells (16, 25). One unit of leukotoxin is defined as the dilution of filtrate sample determined to yield 50% cytotoxicity (14). The leukotoxin-neutralizing capacity of the various antibodies was determined with the same assay except that a preselected dilution of leukotoxin was incubated with antibody dilutions for 1 h at 4°C prior to addition to the BL-3 cells.

Mab production. Eight-week-old female BALB/c mice (Harlan Sprague-Dawley, Indianapolis, Ind.) were immunized with the 102-kDa leukotoxin band (26) excised from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Briefly, 2 mg of protein from the concentrated culture filtrate was stacked at a constant 100 mA, followed by electrophoresis at a constant 120 mA through the separating gel for approximately 5 h. SDS-PAGE was performed as described by Laemmli (23) with a Protean Gel apparatus (Bio-Rad Laboratories, Richmond, Calif.). Stacking and separating gels consisting of 5 and 7% acrylamide (acrylamide-bisacrylamide, 30:0.8), respectively, were used to separate the leukotoxin-containing filtrate. Following electrophoresis, the 102-kDa protein was visualized by light Coomassie blue staining (18), excised, lyophilized, mascerated, and suspended in 12 ml of 0.1 M sodium phosphate-buffered physiological saline (pH 7.2; PBS). Mice were immunized by intraperitoneal injection of 0.4 ml of the suspension and boosted at 4 weeks with an identical injection. Mice with detectable antileukotoxin titers in the enzyme-linked immunosorbent assay (ELISA) were given a third injection after 2 weeks, and 72 h later, splenocytes were fused with Sp2/0-Ag14 cells (American Type Culture Collection) as described by Fazekas de St. Groth and Scheidegger (10). Hybridomas selected by positive ELISA screening with crude concentrated filtrate were cloned by limiting dilution, and antibody isotypes were determined with the immunoselective Isotyping System (Bio-Rad). Ascites were generated for each positive clone (18), and the immunoglobulin G (IgG) was purified from the ascites by using immobilized protein G (Gamma Bind Plus Pre Pack; Genex Corporation, Gaithersburg, Md.). All protein concentrations were determined with a Bradford method kit (Bio-Rad). Rabbit antiserum specific for leukotoxin was prepared by subcutaneous injection of New Zealand White rabbits with the electrophoretically purified leukotoxin described above at monthly intervals.

ELISA. Both direct and capture ELISAs were used. For the direct assay, concentrated crude filtrate, diluted to 1 μg of protein per well in carbonate buffer (0.01 M, pH 9.6), was affixed to the wells of 96-well Immulon II plates (Dynatech, Chantilly, Va.) overnight at 4°C . Unreactive sites were blocked with 3% bovine serum albumin (Sigma) in PBS (PBS-BSA). Culture media and purified ascites IgG were diluted in PBS containing 0.05% Tween-20 (Sigma; PBS-Tween) and incubated in the ELISA wells for 1 h at 37°C . The wells were washed three times with PBS-Tween and then incubated with protein G-horseradish peroxidase (HRP) (Genex) at 37°C for 1 h. The wells were washed with PBS-Tween, and bound antibody-protein G-HRP was detected with ABTS [2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid)] and H_2O_2 (Sigma) in an automated Microplate reader (Bio-Tek, Burlington, Vt.).

Western immunoblot. SDS-PAGE of samples was performed in a Mini Protean II Gel Apparatus (Bio-Rad) with precast 4 to 15% gradient gels (Bio-Rad). Concentrated leukotoxin filtrate protein (2 μg per lane) was electrophoresed for 90 min with constant amperage of 14 mA and transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore) with a semidry protein apparatus (Gelman, Ann Arbor, Mich.) in 0.025 M Tris buffer (pH 8.2) containing 0.19 M glycine and 0.1 M SDS. The membranes were washed in Tris-buffered saline (TBS), immersed in 3% gelatin (Bio-Rad) in TBS, and incubated at 37°C for 1 h to block residual protein-binding sites. The membranes were washed in deionized H_2O and then in TBS with 0.05% Tween-20 (TBS-Tween) and incubated with antibody overnight at room temperature. Bound antibody was detected, following deionized H_2O and TBS-Tween washes, with protein G-HRP (Genex) followed by 4-chloro-1-naphthol and 30% H_2O_2 (Sigma). Development was terminated by washing the membranes with several changes of deionized H_2O . Transferred protein was stained on PVDF membranes by the India ink method of Hancock and Tsang (17).

Flow cytometry. Binding of cells by antibodies was performed as described by Harlow and Lane (18). Briefly, concentrated A1 and A2 culture filtrates were preincubated for 50 min at 4°C with 20 μg of MAb Ltx-2 in a total volume of 100 μl containing 100 U of leukotoxin. BL-3 cells (10^6 in 35 μl of PBS) were then added, and the suspension was incubated for an additional 30 min at 37°C . Following three cycles of low-speed centrifugation and washing with cold PBS containing 3% BSA and 0.02% sodium azide, the cells were treated with rabbit antileukotoxin antibody for 1 h at 4°C and washed three times as described above. To detect cell-bound rabbit antileukotoxin antibodies, the cells were treated with fluorescein-conjugated donkey anti-rabbit immunoglobulin antibody (Jackson Laboratories, West Grove, Pa.). After a wash, single-color fluorescence analysis was performed with a FACScan fluorescence-activated cell sorter (Becton Dickinson, San Jose, Calif.).

Plasmid manipulations. Plasmids containing internal deletions of the *lktA* gene were derived from pYFC19 (4, 8), in which the *lktA* genes are expressed under *lacPO* control. The methods for generating the deletion alleles carried by pDS14 (codons 34 to 378), pWC25 (codons 246 to 357), pDS25 (codons 358 to 548), and pDS27 (codons 549 to 768) have been described previously (8). The carboxyl-terminal truncation carried by pWC64 was generated by introducing a universal translational terminator (Pharmacia) into the *Cla*I site of pYFC19. The leukotoxin deletion protein expressed from pWC64 is missing the carboxyl-terminal 185 residues of the native protein. Deleted forms of the LktA protein were

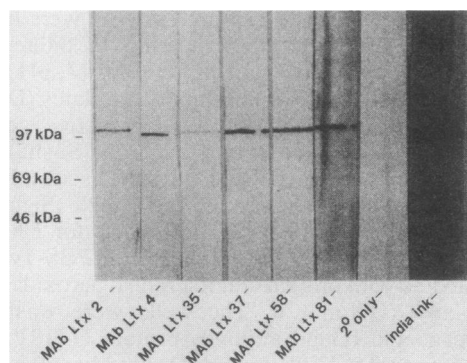


FIG. 1. Immunoblot of MABs reacting with SDS-PAGE of concentrated A1 leukotoxin filtrate transferred to PVDF membrane. Total proteins are visualized in the India ink lane, and the 2° only lane is a control without primary antibody.

prepared by growing *Escherichia coli* TB1 harboring the various plasmids in BHI broth to an OD₅₅₀ of >2.0. Six-milliliter late-logarithmic-phase cultures were washed and precipitated with 10% trichloroacetic acid (TCA) (8). The total cellular protein was washed once with methanol to remove TCA and boiled in 0.5 ml of SDS-PAGE sample buffer, and 25 µl of sample was processed for Western blotting.

CNBr cleavage of leukotoxin. CNBr cleavage was performed as described by Changchien et al. (5) and Oropeza-Wekerle et al. (30). Briefly, 150 mg of CNBr (Sigma) was dissolved in 1.5 ml of 70% formic acid. Then, 0.5 ml of this mixture was added to 18 µg of lyophilized leukotoxin filtrate protein and incubated at room temperature for 48 h. An equal amount of deionized H₂O was added to this mixture, which was then lyophilized. The lyophilized product was rehydrated with a minimal volume of deionized H₂O and subjected to SDS-PAGE.

RESULTS

MAB characterization. Six hybridoma clones producing MABs reactive by ELISA with concentrated leukotoxin-containing filtrate were derived from mice immunized with electrophoretically purified leukotoxin. All six MABs reacted with a protein band at approximately 102 kDa upon Western immunoblot following SDS-PAGE of the crude concentrated leukotoxin (Fig. 1); however, only Ltx-2, an IgG1 MAB, displayed neutralizing activity for the leukotoxin. With sensitive BL-3 target cells, Ltx-2 effected 50% neutralization of 3 and 1 U of leukotoxin activity at 300 and 30 ng/ml, respectively (Fig. 2).

Epitope distribution among serotype leukotoxins. Leukotoxin production is a characteristic of all *P. haemolytica* serotypes (21, 32). Therefore, MAB Ltx-2 was tested for neutralization of the leukotoxin produced by other *P. haemolytica* serotypes. Culture filtrates from isolates representing serotypes 1 through 12 were adjusted to 1 and 4 U and incubated with Ltx-2 at 25 µg/ml, a concentration of the antibody at least 100-fold greater than required to neutralize 3 U of A1 toxin (Fig. 2). The results are given in Table 1. MAB Ltx-2 neutralized leukotoxic activity derived from serotypes 1, 5, 6, 7, 8, 9, and 12 at both 4 and 1 U. However, the MAB did not neutralize the leukotoxic activity produced by serotypes 2, 3, 4, and 10. All of the leukotoxins neutralized were produced by serotypes of biotype A (serotypes 1,

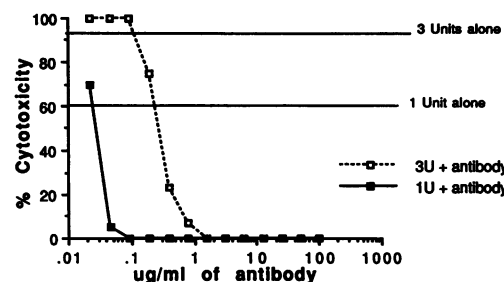


FIG. 2. MAB Ltx-2 neutralization of A1 leukotoxin. Serial dilutions of MAB were preincubated with either 3 or 1 U of leukotoxin, and inhibition of cytotoxicity for BL-3 cells was determined by the neutral red cytotoxicity assay.

5, 6, 7, 8, 9, and 12). A2 leukotoxin was the only A biotype toxin that was not neutralized by MAB Ltx-2. The remaining leukotoxins that were not neutralized were produced by serotypes classified in biotype T (T3, T4, and T10). The serotype 11 strain tested did not produce leukotoxin and is not shown in the table.

Further testing of MAB Ltx-2 revealed that the MAB did not react by ELISA with A2 *P. haemolytica* leukotoxin and reacted too faintly in the Western immunoblot for photographic reproduction. In addition, when Ltx-2 reactivity with T3, T4, and T10 leukotoxins was assessed by Western immunoblot, reactivity was detected only with the T10 leukotoxin band (not shown).

Effect of MAB Ltx-2 on leukotoxin-target cell interaction. MAB Ltx-2 could effect toxin neutralization either by influencing a critical function or site involved in pore formation or by preventing association of the toxin with the target cell. Flow cytometry was performed to determine whether Ltx-2 acted to block the association of A1 leukotoxin with the BL-3 cells. Leukotoxin was incubated with and without Ltx-2 and then added to the target cells. Leukotoxin associated with the target cells was detected by adding rabbit antisera raised against electrophoretically purified leukotoxin followed by fluorescein isothiocyanate-labeled anti-

TABLE 1. Neutralization of leukotoxin activity from log-phase culture filtrates of *P. haemolytica* serotypes^a

<i>P. haemolytica</i> serotype	% Cytotoxicity ^b (mean ± SE, n = 3)			
	1 U	1 U + Ab	4 U	4 U + Ab
1	43 ± 3	0 ± 1	97 ± 1	12 ± 3
2	65 ± 8	<u>67 ± 1</u>	96 ± 1	<u>97 ± 0</u>
2	69 ± 3	<u>71 ± 4</u>	90 ± 4	<u>92 ± 3</u>
3	30 ± 3	<u>34 ± 5</u>	85 ± 3	<u>76 ± 4</u>
4	60 ± 3	<u>70 ± 4</u>	98 ± 1	<u>99 ± 1</u>
5	55 ± 2	3 ± 5	100 ± 0	0 ± 2
6	40 ± 7	0 ± 4	99 ± 1	0 ± 4
7	63 ± 2	0 ± 3	100 ± 0	0 ± 3
8	60 ± 6	0 ± 3	100 ± 3	0 ± 3
9	50 ± 5	0 ± 3	100 ± 3	0 ± 1
10	42 ± 5	<u>48 ± 7</u>	87 ± 1	<u>86 ± 1</u>
10 (ATCC)	45 ± 5	<u>60 ± 5</u>	92 ± 1	<u>85 ± 2</u>
12	50 ± 8	0 ± 5	95 ± 5	0 ± 3

^a All strains were from the collection of G. Frank unless denoted ATCC (American Type Culture Collection).

^b Percent cytotoxicity for BL-3 cells was determined by uptake of neutral red. The amount (units) of leukotoxin or leukotoxin preincubated with MAB Ltx-2 at 25 µg/ml (Ab) is indicated. Underlined values represent cytotoxicity that was not neutralized by MAB Ltx-2.

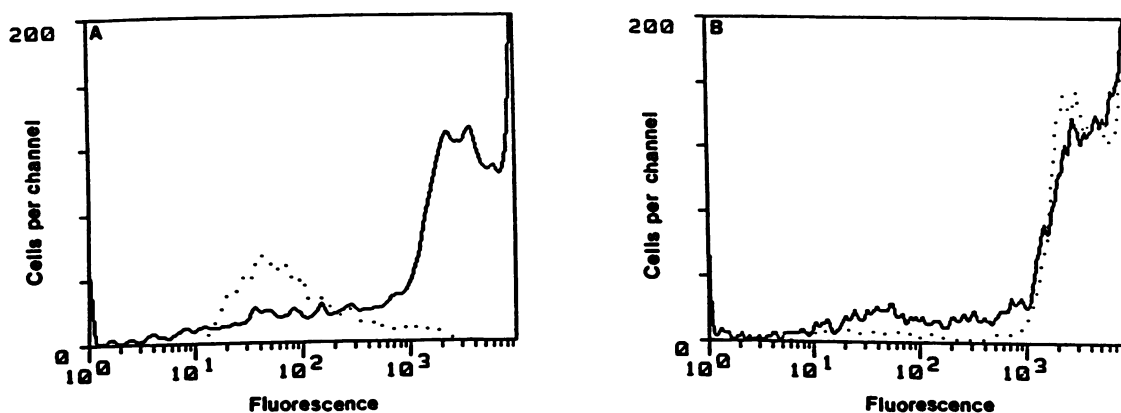


FIG. 3. Flow cytometry detecting A1 or A2 leukotoxin associated with BL-3 target cells. Leukotoxin was incubated with MAb-Ltx-2 prior to addition to BL-3 cells. Following addition to BL-3 cells, cell-bound leukotoxin was detected by using rabbit antileukotoxin followed by FITC-labeled donkey anti-rabbit immunoglobulin antisera. Solid lines (—) represent leukotoxins without MAb Ltx-2 preincubation. Dotted lines (· · ·) represent leukotoxins preincubated with the MAb. (A) Binding of A1 leukotoxin; (B) binding of A2 leukotoxin.

rabbit immunoglobulin antisera. Preincubation of leukotoxin with MAb Ltx-2 blocked its association with the target cells (Fig. 3A). Fifty percent of the BL-3 cells were positive for bound leukotoxin. Preincubation of the toxin with Ltx-2 reduced this percentage to essentially 0, which was not different from the FITC-labeled control. As an additional control, the same experiment was performed with A2 leukotoxin. The detecting rabbit anti-A1 leukotoxin antiserum effectively neutralized A2 leukotoxin in addition to A1 (not shown). MAb Ltx-2 did not prevent the association of A2 leukotoxin with BL-3 cells (Fig. 3B). Without Ltx-2, 52% of the BL-3 cells were positive for bound A2 leukotoxin. Preincubation of the toxin with Ltx-2 did not reduce this value; approximately 60% of the cells were positive for bound A2 leukotoxin. Thus, A1 leukotoxin, which is neutralized by the MAb, was prevented from associating with the target cells, while the MAb did not prevent the binding of or neutralize the A2 leukotoxin.

Preliminary epitope mapping. CNBr cleavage fragments and mutant deletion proteins of the leukotoxin were used to provide preliminary generalized localization of the epitope recognized by MAb Ltx-2. The native leukotoxin contains five methionine residues at amino acid positions 1, 156, 408,

449, and 939 (26). Following complete CNBr cleavage, only one band was detected by immunoblot after SDS-PAGE. This band migrated at about 50 kDa, approximately the mass of an expected 490-amino-acid cleavage fragment containing amino acids 450 to 939 (Fig. 4).

The derivatives of pYFC19 with deletions of the *lktA* gene are presented in Fig. 5. These mutated genes in the *E. coli* host, TB1, produced truncated proteins with deletions across virtually the entire protein. MAb Ltx-2 immunoblots of SDS-PAGE gels of 10% TCA-precipitated intact cells revealed single bands for all the truncated proteins except pWC64, which had a deletion of the carboxy terminus beginning at amino acid 768. Although not shown in the immunoblot (Fig. 4), this truncated protein was visualized by both rabbit and bovine antileukotoxin antisera (8) and was detectable by India ink stain. The weakly stained band for pDS25 was also weakly visualized by rabbit and bovine antileukotoxin antisera, indicating lesser production of this mutant protein.

Results with the deletion proteins confirmed the CNBr localization of Ltx-2 reactivity within the carboxyl half of the native protein and suggested possible localization of an epitope between amino acids 768 and 939. However, this indirect approach was not definitive, and the possibility of multiple epitopes between amino acids 450 and 768, whose

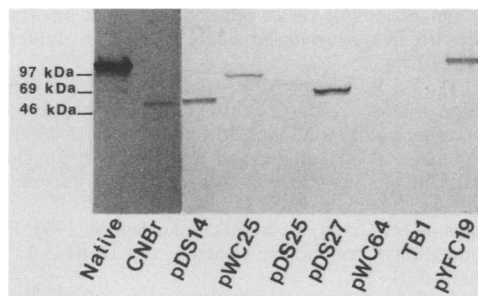


FIG. 4. Immunoblots of CNBr cleavage fragments and A1 leukotoxin deletion proteins. Lyophilized culture filtrate was cleaved with CNBr dissolved in 70% formic acid, and SDS-PAGE and immunoblotting with MAb-Ltx-2 were performed following lyophilization and resuspension in distilled H₂O. The deletion protein samples were TCA precipitates of *E. coli* TB1 harboring the various plasmids. Lane 8 is the precipitate from the *E. coli* host harboring pHG165.

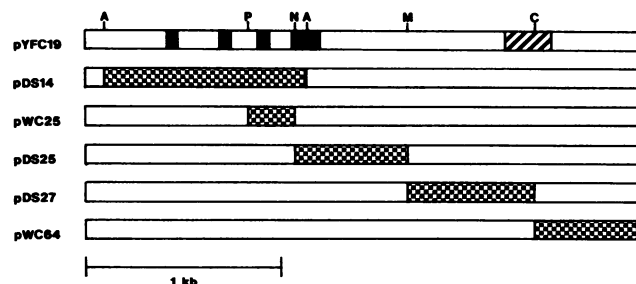


FIG. 5. Deletion maps of mutant *lktA* genes derived from pYFC19. Deleted segments are represented by checkered blocks. Restriction enzyme sites in pYFC19 are: A, *NaeI*; P, *PvuII*; N, *NsiI*; M, *MluI*; and C, *ClaI*. The solid boxes in pYFC19 represent potential membrane-spanning regions, and the glycine-aspartic acid repeat domain is represented by the hatched box.

recognition by Ltx-2 is influenced by the 768 to 939 sequence, cannot be excluded.

DISCUSSION

Shewen and Wilkie (32) prepared specific antisera against serotypes 1 through 12 of *P. haemolytica* and reported various degrees of leukotoxin cross-neutralization. Type-specific rabbit serum neutralized homologous toxin and, to a degree, heterologous toxin. These implied antigenic differences between serotype toxins are further supported by the present study. MAb Ltx-2 neutralized leukotoxin from most, but not all, of the 12 serotypes tested. The MAb neutralized all but one of the A biotype leukotoxins but failed to neutralize leukotoxin from any of the three T biotypes. Furthermore, by Western immunoblot, Ltx-2 reacted only with one of the three T biotype toxins, T10. Although the present findings can be regarded only as preliminary, the results obtained with this MAb indicate that the epitope is either missing in T biotype leukotoxin or is not critical for leukotoxic activity. While definitive proof will require testing of additional T biotype isolates and eventual cloning and sequencing of T biotype *lktA*, the results suggest that sequence and/or conformational differences exist between the leukotoxins from A and T biotypes in addition to differential fermentation of arabinose and trehalose, the current discriminator between the two biotypes (2).

It is additionally interesting that the epitope for MAb Ltx-2 was not definitively demonstrable in A2 leukotoxin. A1 and A2 isolates predominate as causes of pneumonia in domestic ruminants. A1 is the most frequent agent associated with bovine disease, while A2 is the most frequent isolate from sheep (12, 15). Again, definite proof will require testing of additional A2 isolates and eventual cloning and sequencing of A2 *lktA*; however, this observation is suggestive of sequence or conformational differences between A1 and A2 leukotoxin as well as between A and T leukotoxin. This potential difference is further strengthened by the failure of additional A1-derived MABs to react with A2 leukotoxin. By ELISA, only one of the nonneutralizing MABs, Ltx-81, clearly reacted with the A2 leukotoxin. Although the majority of nonneutralizing MABs reacted with denatured A2 leukotoxin by Western blot analysis, MAB Ltx-35 failed to react even under these conditions. Thus, at least two A1 epitopes could not be definitively detected in A2 leukotoxin.

The nature of the epitope recognized by MAb Ltx-2 is unclear. The MAB reacted weakly with toxin that had been affixed to ELISA wells in alkaline carbonate buffer. Ltx-2, however, was a very potent neutralizer of soluble toxin, and although the titration is not presented in the Results section, Ltx-2 reacted very well by ELISA when the leukotoxin was trapped by other toxin-specific MABs. Apparently, the epitope recognized by the MAB is more accessible when the leukotoxin is in soluble form rather than affixed directly to the ELISA well. Recognition of the epitope by immunoblot following the denaturing conditions of SDS-PAGE suggests that the epitope is continuous rather than discontinuous.

The mechanism by which the leukotoxin associates with target cell membranes has not been elucidated. The Ca²⁺-binding glycine-rich repeat domain is critical for RTX toxin effects and has been proposed to function in binding (24, 27). However, it remains unclear whether this requirement is important in initial recognition or at a secondary stage. Regardless, binding and pore formation appear to be separable events (9, 27), and evidence reported by Cruz et al. (8)

indicates that the binding functions are distinct from the lytic functions of A1 leukotoxin. Mutant toxins with whole or partial deletions of the putative membrane-spanning regions agglutinated BL-3 cells and, in addition, were antagonistic for cell lysis by native leukotoxin. In the present study, MAb Ltx-2 prevented the leukotoxin from associating with the target cells, as measured by flow cytometry, suggesting that the MAB neutralizes the toxin by preventing its initial association with the target cell membrane. Thus, the MAB may react with an epitope associated with the binding domain of the leukotoxin. Conversely, Ltx-2 binding may prevent association of the leukotoxin by interfering with transitional-state changes or even by enhancing leukotoxin inactivation. The correct interpretation obviously will require further investigation; however, it is interesting that preliminary mapping positions the epitope in an amino acid sequence devoid of potential membrane-spanning regions but containing at least the last two repeats in the glycine-rich repeat domain. Work is in progress to localize this potentially important epitope more precisely.

ACKNOWLEDGMENTS

This work was supported in part by the Center of Excellence in Livestock Diseases and Human Health, College of Veterinary Medicine, University of Tennessee, Knoxville, and by USDA grant 88-37266-3926 (D.K.S.).

REFERENCES

1. Adlam, C. 1989. The structure, function and properties of cellular and extracellular components of *Pasteurella haemolytica*, p. 75–92. In C. Adlam and J. M. Putter (ed.), *Pasteurella* and pasteurellosis. Academic Press, London.
2. Biberstein, E. L. 1978. Biotyping and serotyping of *Pasteurella haemolytica*, p. 253–269. In T. Bergan and J. R. Norris (ed.), *Methods in Microbiology*. Academic Press, Inc., New York.
3. Boehm, D. F., R. A. Welch, and I. S. Snyder. 1990. Domains of *Escherichia coli* hemolysin (HlyA) involved in binding of calcium and erythrocyte membranes. *Infect. Immun.* 58:1959–1964.
4. Chang, Y. F., R. Y. Young, D. Post, and D. K. Struck. 1987. Identification and characterization of the *Pasteurella haemolytica* leukotoxin. *Infect. Immun.* 55:2348–2354.
5. Changchien, L., R. Conrad, and G. R. Craven. 1988. Isolation of fragments of ribosomal proteins that recognize rRNA. *Methods Enzymol.* 164:258–270.
6. Clinkenbeard, K. D., D. A. Mosier, A. L. Timko, and A. W. Confer. 1989. Effects of *Pasteurella haemolytica* leukotoxin on cultured bovine lymphoma cells. *Am. J. Vet. Res.* 50:271–275.
7. Conlon, J. A., P. A. Shewen, and R. Y. C. Lo. 1991. Efficacy of recombinant leukotoxin in protection against pneumonic challenge with live *Pasteurella haemolytica* A1. *Infect. Immun.* 59:587–591.
8. Cruz, W. T., R. Young, Y. F. Chang, and D. K. Struck. 1990. Deletion analysis resolves cell-binding and lytic domains of the *Pasteurella* leukotoxin. *Mol. Microbiol.* 4:1933–1939.
9. Eberspacher, B., F. Hugo, and S. Bhakdi. 1989. Quantitative study of the binding and hemolytic efficiency of *Escherichia coli* hemolysin. *Infect. Immun.* 57:983–988.
10. Fazekas de St. Groth, S., and D. Scheidegger. 1980. Production of monoclonal antibodies: strategy and tactics. *J. Immunol. Methods* 35:1–21.
11. Felmlee, T., and R. A. Welch. 1988. Alterations of amino acid repeats in the *Escherichia coli* hemolysin affect cytolytic activity and secretion. *Proc. Natl. Acad. Sci. USA* 85:5269–5273.
12. Frank, G. H. 1989. Pasteurellosis of cattle, p. 197–222. In C. Adlam and J. M. Rutter (ed.), *Pasteurella* and pasteurellosis. Academic Press, London.
13. Gentry, M. J., A. W. Confer, and R. J. Panciera. 1985. Serum neutralization of cytotoxin from *Pasteurella haemolytica* serotype 1 and resistance to experimental bovine pneumonic pas-

- teurellosis. Vet. Immunol. Immunopathol. 9:239-250.
14. Gerbig, D. G., Jr., R. D. Walker, J. C. Baker, J. S. Foster, and R. N. Moore. 1989. Calcium ion involvement in the action of *Pasteurella haemolytica* leukotoxin. Vet. Microbiol. 19:325-335.
 15. Gilmour, N. J. L., and J. S. Gilmour. 1989. Pasteurellosis of sheep, p. 223-252. In C. Adlam and J. M. Rutter (ed.), *Pasteurella* and pasteurellosis. Academic Press, London.
 16. Greer, C. N., and P. E. Shewen. 1986. Automated colorimetric assay for the detection of *Pasteurella haemolytica* leukotoxin. Vet. Microbiol. 12:33-42.
 17. Hancock, K., and V. C. W. Tsang. 1983. India ink staining of proteins on nitrocellulose paper. Anal. Biochem. 133:157-162.
 18. Harlow, E., and D. Lane. 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 19. Henry, C. W. 1984. Shipping fever pneumonia: a new look at an old enemy. Vet. Med. 79:1200-1206.
 20. Highlander, S. K., M. Chidambaram, M. J. Engler, and G. M. Weinstock. 1989. DNA sequence of the *Pasteurella haemolytica* leukotoxin gene cluster. DNA 8:15-28.
 21. Himmel, M. E., M. D. Yates, L. H. Lauerma, and P. G. Squire. 1982. Purification and partial characterization of a macrophage cytotoxin from *Pasteurella haemolytica*. Am. J. Vet. Res. 43:764-767.
 22. Ji, G. E., and P. O'Hanley. 1990. Epitopes of *Escherichia coli* alpha-hemolysin: identification of monoclonal antibodies that prevent hemolysis. Infect. Immun. 58:3029-3035.
 23. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.
 24. Lo, R. Y. C. 1990. Molecular characterization of cytotoxins produced by *Haemophilus*, *Actinobacillus*, and *Pasteurella*. Can. J. Vet. Res. 54:533-535.
 25. Lo, R. Y. C., P. A. Shewen, C. A. Strathdee, and C. N. Greer. 1985. Cloning and expression of the leukotoxin gene of *Pasteurella haemolytica* A1 in *Escherichia coli* K-12. Infect. Immun. 50:667-671.
 26. Lo, R. Y. C., C. A. Strathdee, and P. E. Shewen. 1987. Nucleotide sequence of the leukotoxin genes of *Pasteurella haemolytica* A1. Infect. Immun. 55:1987-1996.
 27. Ludwig, A., T. Jarchau, R. Benz, and W. Goebel. 1988. The repeat domain of *Escherichia coli* haemolysin (HlyA) is responsible for its calcium-dependent binding to erythrocytes. Mol. Gen. Genet. 214:553-561.
 28. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 29. Moore, R. N., R. D. Walker, G. A. Shaw, F. M. Hopkins, and E. P. Shull. 1985. Antileukotoxin antibody produced in the bovine lung after aerosol exposure to viable *Pasteurella haemolytica*. Am. J. Vet. Res. 46:1949-1952.
 30. Oropeza-Wekerle, R. L., P. Kern, D. Sun, S. Muller, J. P. Briand, and W. Goebel. 1991. Characterization of monoclonal antibodies against alpha-hemolysin of *Escherichia coli*. Infect. Immun. 59:1846-1852.
 31. Pellett, S., D. F. Boehm, I. S. Snyder, E. Rowe, and R. A. Welch. 1990. Characterization of monoclonal antibodies against *Escherichia coli* hemolysin. Infect. Immun. 58:822-827.
 32. Shewen, P. E., and B. N. Wilkie. 1983. *Pasteurella haemolytica* cytotoxin: production by recognized serotypes and neutralization by type-specific rabbit antisera. Am. J. Vet. Res. 44:715-719.
 33. Shewen, P. E., and B. N. Wilkie. 1985. Evidence for the *Pasteurella haemolytica* cytotoxin as a product of actively growing bacteria. Am. J. Vet. Res. 46:1212-1214.
 34. Slocumbe, R. F., J. Malark, R. Ingersoll, F. J. Derksen, and N. E. Robinson. 1985. Importance of neutrophils in the pathogenesis of acute pneumonic pasteurellosis in calves. Am. J. Vet. Res. 46:2253-2258.
 35. Strathdee, C. A., and R. Y. C. Lo. 1989. Cloning, nucleotide sequence, and characterization of genes encoding the secretion function of the *Pasteurella haemolytica* leukotoxin determinant. J. Bacteriol. 171:916-928.
 36. Walker, R. D., T. W. Shultz, F. M. Hopkins, and M. J. Bryant. 1984. Growth phase dependent phagocytosis of *Pasteurella haemolytica* by bovine pulmonary macrophages. Am. J. Vet. Res. 45:1230-1234.
 37. Welch, R. A., S. Pellet, G. Rowe, K. Swihart, T. Uphoff, D. Boehm, and I. S. Snyder. 1990. Molecular characterization of HlyA and HpmA, the prototypes for two gram negative hemolysin gene families, p. 61-68. In R. Rappuoli et al. (ed.), Bacterial protein toxins. Gustav Fisher, Stuttgart, Germany.